

Amendments to the Specification:

Please replace the paragraph beginning at page 1, line 1 with the following amended paragraph:

This application is a continuation of International Patent Application No. PCT/US99/28826, filed Dec. 3, 1999 and published in English on June 15, 2000 as WO 00/34487, which is a continuation of U.S. Application Serial No. 09/206,898, filed December 7, 1998 (issued as U.S. Patent No. 6,355,255 ~~5,846,547~~), which is a continuation-in-part of U.S. Application Serial No. 08/589,756 filed January 22, 1996 (issued as U.S. Patent No. 6,355,255 ~~5,846,547~~). USSN 08/589,756 is incorporated by reference herein.

Please replace the paragraph beginning at page 8, line 24 with the following amended paragraph:

~~Figure 9~~ Figures 9A and 9B. Serum IgG and secretory IgA responses after intranasal immunization of mice with the purified ΔSCPA49 protein. Serum and saliva levels of SCPA49 specific IgG were determined by indirect ELISA. Sera from each mouse were diluted to 1: 2,560 in PBS; saliva was diluted 1:2 in PBS. Figure 9A shows the sIgA experimental results; Figure 9B shows the IgG experimental results.

Please replace the paragraph beginning at page 8, line 30 with the following amended paragraph:

~~Figure 10~~ Figures 10A and 10B. Comparison of the ability of serotype M49 streptococci to colonize immunized and non-immunized CD1 female mice. Each experimental group contained 13 mice which were infected intranasally (i.n.) with 2.0×10^8 CFU. The data were analyzed statistically by the χ^2 test. Figures 10A and 10B show the results of the repeated experiment.

Please replace the paragraph beginning at page 10, line 24 with the following amended paragraph:

The ~~catalytic domain~~ or active site of SCP is composed of the charge transfer system and the specificity crevice. The charge transfer system, also called the catalytic domain, contains residues Asp¹³⁰, His¹⁹³, Asn²⁹⁵ and Ser⁵¹² (Figs. 1 and 2). A modification, i.e., a deletion, insertion or substitution, of any one of these amino acids will inactivate the enzyme. The specificity crevice, on the other hand, is predicted to be formed by Ser²⁶⁰, Phe²⁶¹, Gly²⁶², Ile⁴¹⁵, Tyr⁴¹⁶ and Asp⁴¹⁷. Modification by substitution of these amino acids could change the substrate specificity of the enzyme or eliminate proteolytic activity altogether. Modification by deletion of these amino acids would also inactivate the enzyme. The catalytic domain depends on the tertiary structure of the protein that is created when the mature enzyme folds into its active state. This domain is not formed from a contiguous linear array of amino acid residues. Alternatively, modification may also reduce binding of variant SCP to the substrate. Binding may be reduced by 50%, 70% or even 80%.

Please replace the paragraph beginning at page 12, line 18 with the following amended paragraph:

The air sac model was modified to compare clearance of wild-type SCP⁺ and SCP⁻ streptococci (i.e., group A streptococci which carried a variant non-functional form of SCP), and to analyze the cellular infiltrate at an early stage of infection. Tissue suspensions were assayed for viable streptococci on blood agar plates and the cellular infiltrate was analyzed by fluorescent cell sorting (FACS). In FACS analysis, individual cells in suspension are labelled with specific fluorescent monoantibodies. Aliquots of labelled cells are injected into a ~~FAC-Scan~~ FACScan™ flowcytometer, or fluorescent cell sorter, which counts cells based on their unique fluorescence. The experiments using the air sac model indicated that streptococci that were SCP⁺ were more virulent than streptococci that were SCP⁻.

Please replace the paragraph beginning at page 23, line 3 with the following amended paragraph:

e) In vitro effects of mutations on SCP. The impact of insertions and deletions on the expression of SCP antigen and peptidase activity was assessed by Western blot and PMNs adherence assays. Streptococci were incubated in 100 ml THY at 37°C overnight. The culture pellet was washed two times in 5 ml cold 0.2 M NaAcetate (pH 5.2), then suspended in 1 ml TE-sucrose buffer (20% sucrose 10 mM Tris, 1 mM EDTA, pH 7.0) and 40 µl Mutanolysin mutanolysin. The mixture was rotated at 37°C for 2 hr, then centrifuged 5 min at 4500 rpm. Supernatants contained protease inhibitor, 100 mM phenylmethyl sulfonyl fluoride (PMSF). Electrophoresis and Western blotting methods were performed as described in Laemmli, U. K., "Cleavage of structural proteins during the assembly of the head of bacteriophage T4," Nature 227:680-685 (1970). The primary antiserum used to detect SCP protein on Western and colony blots was prepared by immunization of a rabbit with purified recombinant SCP protein. Binding was detected by anti-rabbit antibody alkaline phosphatase conjugate.

Please replace the paragraph beginning at page 23, line 17 with the following amended paragraph:

C5a peptidase activity was measured using a PMN adherence assay. Booth, S. A. et al., "Dapsone suppresses integrin-mediated neutrophil adherence function," J. Invest. Dermatol. 98:135-140 (1992). After incubation of C5a (Sigma, St. Louis, MO) with streptococcal extracts or purified protease, residual C5a can activate PMNs to become adherent to BSA coated wells. First, microtiter wells were coated with 0.5% BSA in PBS and incubated for 1 hr at 37°C. Human PMNs were isolated by centrifugation in Ficoll® Hypaque™ solution Ficoll-Hypaque (Sigma, St. Louis, MO). 40 µl of intact streptococci or protein extracts were incubated with 20 µl of 5 µM C5a in 340 µl of PBS with 1% glucose and 0.1% CaCl₂ at 37°C for 45 min. BSA-coated wells were washed with PBS, and resuspended PMNs and residual C5a were added to wells. The mixture was incubated for 45 min at 37°C in 7% CO₂. Finally, wells were washed to remove nonadherent PMNs. Adherent PMNs were stained with crystal violet and the OD_{570nm} was read in an ELISA reader. The optical density is proportional to the amount of residual C5a or inversely proportional to the amount of SCP activity.

Please replace the paragraph beginning at page 32, line 14 with the following amended paragraph:

A PCR fragment which corresponds to a truncated form of the scpA49 gene was cloned from CS101 M49 group A streptococci (Δ SCPA49). This fragment was amplified by PCR using a forward primer beginning at nucleotide 1033 and a reverse primer beginning at nucleotide 3941 (numbering corresponding to that of Chen, C., and Cleary, P., "Complete nucleotide sequence of the streptococcal C5a peptidase gene of *Streptococcus pyogenes*," J. Biol. Chem., 265:3161-3167 (1990)). The fragment was ligated to the thrombin binding site of glutathione transferase gene on the pGex-4T-1 high expression vector from Pharmacia Inc. The plasmid containing the recombinant scpA fragment, designated pJC6, has been deposited in the American Type Culture Collection, 10801 University Boulevard, Manassas, VA 20110-2209 ~~Rockville, MD~~, under the provision of the Budapest Treaty, and assigned ATCC accession number 98225.

Please replace the paragraph beginning at page 32, line 26 with the following amended paragraph:

The Δ SCPA49, a 2908 bp fragment of scpA49, was amplified by PCR using an scpA49 forward primer containing a BamHI recognition sequence (5'-CCCCCGGATCCACCAAAACCCACAACTC-3') (SEQ ID NO:8) and an scpA reverse primer (5'-GAGTGGCCCTCCAATAGC-3') (SEQ ID NO:9). Sequences which code for the signal peptide and membrane anchor regions of the SCPA protein were deleted from the resulting PCR product. PCR products were digested with BamHI and ligated to BamHI and SmaI restriction sites in the thrombin recognition site of the glutathione S-transferase gene on the pGEX-4T-1 high expression vector from Pharmacia Inc. (Piscataway, NJ). The recombinant plasmid was transformed into *E. coli* DH5 α . The Δ SCPA49 fusion protein from one transformant, *E. coli* (pJC6), was purified by affinity chromatography on a glutathione ~~Sepharose~~ Sepharose[®] 4B column. The transferase-SCP fusion protein from one *E. coli* clone was expressed and purified by affinity chromatography on a glutathione ~~Sepharose~~ Sepharose[®] 4b

column. All methods are described by the manufacturer (Pharmacia). The Δ SCPA49 was cleaved from the hybrid protein by thrombin digestion. The thrombin was removed from eluted SCP by chromatography on a benzamidine ~~Sepharose~~ Sepharose[®] 6B column (Pharmacia). Following digestion with thrombin, thrombin was removed by chromatography on a benzamidine-~~Sepharose~~ Sepharose[®] 6B column. Methods of expression and purification are described by the manufacturer. The affinity purified protein was confirmed to be pure Δ SCPA49 by SDS-PAGE and by Western blot. This affinity purified, truncated Δ SCPA49 protein lacked peptidase activity when tested by the PMN adherence assay (described in Example 1 above). Hyperimmune antiserum, directed against purified Δ SCPA49 was prepared in rabbits.

Please replace the paragraph beginning at page 34, line 3 with the following amended paragraph:

c) Sample collection and ELISA. Blood and saliva samples were collected from anesthetized mice after immunization. All sera were tested for the presence of SCPA49 antibodies by ELISA, as previously described. S.P. O'Connor et al., "The Human Antibody Response to Streptococcal C5a Peptidase," *J. Infect. Dis.*, 163, pp. 109-116 (1990). Purified SCPA49 protein was bound to microtiter wells by addition of 500ng of purified protein in 0.05M bicarbonate buffer (pH 9.6). After overnight incubation at 4°C the wells were washed, then blocked with 0.5% BSA in PBS for 1 hour. Salivation was stimulated in mice by injection of 100 μ l of a 0.1% pilocarpine (Sigma) solution subcutaneously. Saliva samples were collected and spun at 14,000 rpm for 5 min in an ~~Eppendorf~~ Eppendorf[®] microcentrifuge. The supernatants were tested for the presence of secretory IgA against Δ SCPA49 protein by ELISA. ELISA titers represent the highest dilution of individual serum and saliva which had an OD₄₀₅ \geq 0.1.

Please replace the paragraph beginning at page 42, line 20 with the following amended paragraph:

The aspartate and asparagine variants were constructed in much the same fashion, using the reverse primers *scpmutrev717* (5' - CAGTGATTGATGCTGGTTTTGATAA- 3') SEQ ID NO:13 and *scpmutrev1214* (5' - AGCTACTATCAGCACCAG - 3') SEQ ID NO:14 to construct 311 bp and 805 bp megaprimers, respectively. The primer *scpmutrev717* was used to generate variant protein SCPA49D130A, and primer *scpmutrev1214* was used to generate variant protein SCPA49N295A (see Table 6 below). After ~~Qiaquick~~ Qiaquick™ purification, however, the megaprimer was treated with 0.1 U mung bean nuclease (per 4 µg DNA) and incubated at 30°C for 10 minutes. The nuclease was removed by phenol/chloroform extraction, and the megaprimer recovered in the aqueous phase by ethanol precipitation. The pellet was resuspended in 80 µl sterile double distilled water, and 37 µl of this was used in each 100 µl asymmetrical PCR reaction. The mutated gene was then cloned into pGEX 4T-1 as previously described. Sequencing of variants was performed using ³⁵S-labeled dATP and the ~~Sequenase~~ Sequenase™ kit (Stratagene Stratagene®) or using automated fluorescent sequencing at the University of Minnesota Microchemical Facility.

Please replace the paragraph beginning at page 44, line 4 with the following amended paragraph:

The resultant PCR product corresponding to bases 940-3954 was cloned into an intermediate vector pCR2.1 (~~Invitrogen~~ Invitrogen™, Inc.) and transformed into E. coli Top10F cells (~~Invitrogen~~ Invitrogen™, Inc.). Plasmid DNA from an appropriate transformant was restricted with EcoRI and BamHI. The 3018 base fragment, containing the fragment of *scpA1*, was gel purified following standard procedures and ligated into the expression vector pTrc99a (Pharmacia) restricted with the same enzymes. This ligation was transformed into E. coli DH5α cells and a transformant was selected that contained the desired plasmid construction. The resultant plasmid places the PCR fragment of *scpA1* behind a Shine-Dalgarno sequence and ATG start site, and is under the transcriptional control of the *trc* Promoter, that is inducible with the allolactose analogue IPTG.

Please replace the paragraph beginning at page 45, line 24 with the following amended paragraph:

These sets of PCR primers were used in three separate reactions. The template DNA was pLP605, which contained the wild-type scpA1 sequence. The PCR products were subsequently self-ligated and transformed into the E. coli strain Top10F' (~~Invitrogen~~ InvitrogenTM, Inc.). Transformants were screened for the appropriate size and restriction pattern. The sequence change in the S512A variant destroys a unique SpeI restriction site so that this mutation could be identified directly by restriction analysis. All potential variants were confirmed by DNA sequencing. Subsequently, the D130A mutation was combined with the S512A mutation to form a double variant utilizing a unique PstI site between these two regions of the protein. The final alteration was to change the antibiotic selection from ampicillin to kanamycin by moving the variant scpA1 genes to a previously altered pTRC99a vector (Pharmacia, Inc.) containing the kanamycin gene.